

11/PRTS

METHOD FOR THE SPECIFIC DETECTION AND IDENTIFICATION OF

RETROVIRAL NUCLEIC ACIDS/RETROVIRUSES IN A SPECIMEN

Description

[0001] The invention relates to a method for the specific detection and identification of retroviral nucleic acids/retroviruses in an arbitrary specimen and a diagnosis kit for implementing this method. It also relates to retrovirus-specific probes for reverse dot blot hybridisation and retrovirus-specific oligonucleotide primer mixtures (MOP) comprising forward and reverse primers for producing amplificates of retrovirus-specific nucleic acids from the specimen.

[0002] Exogenous and endogenous retroviruses (HERV) are etiological agents for a multiplicity of tumorigenic diseases in humans and animals. They are involved in the formation of tumours and leukaemias in numerous animal models but also in humans (HTLV-I and II). Others also cause immunodeficiency diseases (HIV). Present research indicates that retroviruses may also play a role as triggers of autoimmune diseases (Kalden and Herrmann, 1993) and neuronal degenerative diseases such as multiple sclerosis (Tuke et al. 1997). Intensive research in the field of endogenous and exogenous retroviruses has so far led to the continual discovery of new retroviral sequences in the human genotype whose expression could possibly be associated with specific diseases. For example, the expression of Gag proteins of the HERV-K family is associated with almost all forms of testicular and ovarian germinomas (Sauter et al. 1995). Antibodies against HERV-K Env protein were detected in human sera (Vogetseeder et al. 1993). The HERV-K-IDDM env gene which was isolated from patients with type-1 diabetes possibly codes for an endogenous superantigen (Conrad et al. 1997).

[0003] Statistically reliable studies using large groups of patients are required to correlate specific diseases with the activity of specific endogenous or exogenous retroviruses. The expenditure in time and money required for this using the known methods of detection according to the prior art is immense.

[0004] The increasing use of retroviral vector systems in human gene therapy casts doubts on the safety from undesirable side effects (genome

changes in the target cells, transmission of undesirable viruses). Thus, a certain percentage of undesirable gene sections of endogenous or foreign retroviruses are also co-packaged in the retroviral particles for therapeutic application in packaging cell lines (Co-packaging, Sherwin et al. 1987, Scolnick et al. 1979, Takeuchi et al. 1992). For example, transcripts of certain endogenous retroviruses, such as those present in related form in the genome of packaging cell lines, have been detected in retrovirus-like particles (pseudotypes) of the breast cancer cell line T47D (Seifarth et al. 1995, 1998). The packaging of such undesirable retroviral sequences can lead to the recombination and formation of new retroviruses having modified, possible pathogenic properties. The re-integration of such recombinant retroviruses in the genome of the target cells can lead to insertion mutagenesis and consequently to inactivation of important genes of the cell cycle and possibly to tumorigenesis.

[0005] For this reason it is necessary to conduct quality control of the gene vector preparations to be used for gene therapy using a sensitive test system. This could prevent any undesirable retroviral sequences from being transfused. In the event of positive detection, a vector preparation could be subjected to suitable purification (purging) before being administered to the patient. The methods known according to the prior art are not suitable for such an application.

[0006] A controversial issue at the present time is the use of animal organs for transplantation in humans (xenotransplantation). For example, as a result of the shortage of suitable donors, heart valves from pigs are being increasingly transplanted in humans. The transplantation of heart, liver and kidney transplants is also planned. However, recent research has shown that within the transplantation framework and the associated immunosuppression by medication, endogenous or exogenous retroviruses so far suppressed in the donor organ can become activated in the recipient. As has already been demonstrated experimentally, these retroviruses of animal origin are pathogenic for certain human cell types (xenotropism) and could thus lead to a serious systemic disease of the organ recipient. In cases where pathogenic infectious virus particles are formed, transmission to uninvolved third parties (epidemic) cannot be excluded. Last but not least, recombinations of retroviruses of animal origin with endogenous human retroviruses could result in new pathogenic virus recombinants with completely new host tropisms.

[0007] There is thus a need for fast, reliable and at the same time favourably priced detection systems which could be used to test the transplant carrier for infection with retroviruses of animal origin on a regular basis.

[0008] A number of methods for direct and indirect virus detection are available for the detection of viral infections in the prior art. For the direct detection of virus particles, products of viral replication (viral antigens) or an immune response directed against the virus (antiviral antibodies) these include electron microscopy (EM), staining of viral proteins with fluorescent antibodies, "enzyme-linked immunosorbent assay" (ELISA) and radioimmunoassay (RIA). Molecular biological methods such as nucleic acid hybridisations with virus-specific gene probes (dot-blot, southern-blot, northern-blot) and polymerase chain reaction (PCR) with virus-specific primers are being conducted increasingly to directly detect the virus and its nucleic acids.

[0009] In the indirect methods it is usually not the viruses themselves but their after-effects that are detected, i.e. the changes (cytopathic effects) in cells induced by a virus replication. This must usually be conducted in an *in vitro* cell culture system tailored to the virus to be detected. This requires living cells in which the virus to be detected can replicate. Depending on the type of virus, cell cultures, organ cultures, embryonated chick eggs or even laboratory animals are required for the detection. The manifestation of the cytopathic effect (cell lysis, focal or diffuse cell growth, syncytium formation, rounding) and the host spectrum of the virus are used as indices to identify the virus. Frequently however, a precise identification can only be made in combination with serological or molecular biological methods (PCR).

[0010] The relatively low sensitivity of some direct methods of detection (EM, antibody staining) means that the specimen must contain a certain quantity of virus for a successful detection or it must be enriched by suitable methods (ultracentrifuging). If this is not practicable, the virus must be preliminarily cultured in a special *in vitro* cell culture system. Since many viruses possess special host cell tropisms, a special test system is required for each virus to be tested. This results in high laboratory costs, their evaluation is very time-consuming in some cases and requires very great experience.

[0011] Serological methods (ELISA, RIA) are generally highly sensitive and have developed into the current gold standard in virus diagnosis. However, the disadvantage of all serological methods is that a specific antibody is required for each virus to be tested. In one test run the sample to be studied can thus only be tested for one putative virus. Studies of entire expression patterns using these methods can only be made at great expense in time and cost.

[0012] Developments in the field of molecular biology have led to the development of new methods of detection (hybridisations, PCR) which possess similar sensitivity to serological antigen methods of detection. In this case also, the detection success stands or falls by the availability of virus-specific gene probes (hybridisation) or oligonucleotides (PCR). Since the use of several probes or PCR primers is limited because of non-specific interactions in a reaction formulation, many experiments must be conducted in parallel to detect complex expression patterns.

[0013] In view of the circumstances described previously, the problem for the present invention was to provide an efficient and reliable, and at the same time fast method for the multiple detection of endogenous and exogenous retroviruses of human and animal origin.

[0014] This problem is solved using a method of the type specified initially which is characterised in that it comprises the following measures:

[0015] Isolation of nucleic acids, namely DNA and/or RNA from the specimen,

- carrying out a PCR using the isolated DNA or an RT-PCR using the isolated RNA using one or both the primer mixtures MOP-ABD and MOP-C described hereinafter, each consisting of forward primers and reverse primers, whose forward and reverse primers are degenerated oligonucleotides having the nucleotide sequences reproduced in the sequence protocols SEQ ID NO.1 to NO.4 according to the IUPAC nomenclature and a so-called "head" at the 5'-end of these nucleotide sequences, whereby the forward primers of the MOP-ABD mixture exhibit the nucleotide sequences in accordance with SEQ ID NO.1,

namely the nucleotide sequences: "head"-ARAGTNYTDYCHCMRGGH, with 3456 degenerations,

[0016] the reverse primers of the MOP-ABD mixture exhibit the nucleotide sequences in accordance with SEQ ID NO.2, namely the nucleotide sequences: "head"-NWDDMKDTYATCMAYRWA, with 27648 degenerations,

[0017] the forward primers of the MOP-C mixture exhibit the nucleotide sequences in accordance with SEQ ID NO.3, namely the nucleotide sequences: "head"- TKKAMMSKVYTRCYHCARGGG, with 3072 degenerations, and

[0018] the reverse primers of the MOP-C mixture exhibit the nucleotide sequences in accordance with SEQ ID NO.4, namely the nucleotide sequences: "head"-MDVHDRBMDKYMAYVYAHKKA, with 8192 degenerations,

[0019] whereby "head" stands for a nucleotide sequence which comprises an interface for a restriction enzyme and a so-called clamp sequence (for stabilisation of the interface sequence) at the 5' end of this interface,

- purging the (RT)-PCR amplificates obtained and using these in an RDBH method using immobilised RDBH probes which each (per probe) comprise synthetic oligonucleotides whose nucleotide sequence corresponds to the retroviral nucleotide sequence of the retrovirus-specific reverse transcriptase gene of the virus type to be detected with the relevant dot or a section of such a retroviral nucleotide sequence and shows no overlapping with the nucleotide sequences of the forward primers and reverse primers used in the PCR or RT-PCR.

[0020] The object of the present invention is thus a method for detecting retroviral nucleic acids in a sample whereby first all nucleic acids (RNA and DNA) are extracted from the specimen using common methods known to the specialist. Here a distinction is made between DNA and RNA. The isolation of genomic DNA is sufficient to detect retroviruses (proviruses) already integrated in the host cell genome. If the activatability of so far inactive retroviruses, the transcription activity of endogenous retroviruses or the identity of retroviral

particles are to be studied, polyadenylate messenger RNA (mRNA) free from genomic DNA must be isolated. If mRNA is used as the starting material, this mRNA must be transcribed into complementary DNA (cDNA) *in vitro* by means of reverse transcriptase and can then be used as matrices for the following PCR. This combination of reverse transcription and PCR is generally described as RT-PCR.

[0021] The isolated nucleic acids are then subjected to a one-step PCR using primer mixtures according to the invention (MOP-ABD, MOP-C) consisting of retrovirus-specific, degenerated oligonucleotides corresponding to the highly conserved regions within the reverse transcriptase gene (RT gene) of all known human retroviruses (MOP, Shih et al. 1989, Donehower et al. 1990). In this PCR all retrovirus-specific 'reverse transcriptase' homologous sequence sections contained in the specimen are amplified. As a result an amplificate mixture of short retroviral DNA fragments is obtained whose composition reflects the frequency of all retroviral nucleotide sequences to be detected in the specimen. The amplificates are either labelled during the PCR reaction or after this reaction, preferably radioactively, but equally well non-radioactively according to choice (e.g. with biotin or digoxigenin). These labelled amplificates are then used as probes in a hybridisation method (RDBH method) using filter membranes or bio-chips with applied retrovirus-specific oligonucleotides as probes.

[0022] The head or extension sequence of the primer oligonucleotides according to the invention consisting of clamp and interface sequence first has the positive effect that it favourably influences the primer matrix binding kinetics so that the PCR products formed in the first PCR cycle are amplified substantially more efficiently in the following cycles. This has the advantage that retroviral matrices are then amplified themselves or can be amplified if the exactly matching primer is not present in the primer mixture. The interface also has the advantage that it facilitates cloning if necessary.

[0023] It basically holds that the length of the head or extension sequence should not exceed half the length of the complete primer nucleotide sequence.

[0024] An important component of the method according to the invention are the RDBH probes used for the reverse dot blot hybridisation (RDBH) which comprise specific quantities of synthetically produced, exactly defined nucleic acid sequences from the reverse transcriptase gene of those retroviruses already characterised against which the specimen is to be tested. These retrovirus-specific RDBH probes are applied to defined fields (dots) of the RDBH support whereby this can be conventionally used filter membranes having dimensions of several centimetres and also so-called bio-chips having dimensions of a few millimetres (micro-array technology). In the case of filter membranes the probes are preferably linked to these supports covalently by UV crosslinking (e.g. using the commercially available UV radiation source 'StratalinkerTM', Stratagene).

[0025] In the case of DNA chips, oligonucleotide probes are synthesised in situ and fixed in precisely defined positions on a solid support using photolithographic methods (similar to engraving) (e.g. by using perforated masks specific areas of the chip are illuminated to activate photosensitive chemical groups). The support, preferably a glass or nylon surface of approximately 1 cm² forms the hybridisation unit. Each hybridisation unit can contain a very large number of different oligonucleotide probes (up to 400,000). As a result many thousand different sequences can be analysed simultaneously. For each probe all sequence alternatives are presented on the chip and a single one must be recognised by the specimen to be tested. The hybridisation of probe and target sequence (in the specimen) is detected by measuring the amplificate marker intensity. The intensity is proportional to the extent of the hybridisation between probe and target sequence. Each target sequence is identified according to its hybridisation position on the chip. The DNA chip technique was developed by FODOR et al. (Science 251, 767-773, 1991) and is known in the prior art (see V. Oeding et al. 1999, *HYGIENE UND MIKROBIOLOGIE* 1/99, pp. 55-57 and G. Ramsay 1998, *NATURE BIOTECHNOLOGIE*, Vol. 16, 1998, pp. 40-44).

[0026] Hybridisation should take place under highly stringent conditions matched to the length of the probes and the hybridised supports (filter membranes or chips) should then be washed under highly stringent conditions. In cases where the PCR amplicates are radioactively labelled, the identity of

the detectable retroviruses can then be identified after exposing the filter membranes on an x-ray film using the signal pattern of the autoradiograms.

[0027] The method according to the invention can be used for the multiple detection and identification of all human and/or animal-specific retroviral nucleic acids/retroviruses known so far in cell cultures, cell culture residues or body samples or other specimens of biological origin. The only prerequisite is that certain genome sections of the retroviruses to be detected, namely the conserved sequence regions of the reverse transcriptase are known with respect to their DNA nucleotide sequence. This prerequisite is satisfied since the corresponding nucleotide sequences of exogenous and endogenous human retroviruses are generally accessible as gene bank data.

[0028] The method according to the invention for the first time opens up the possibility of providing a universal retrovirus detection system with which the entire spectrum (= expression pattern) of all active endogenous and exogenous retroviral nucleotide sequences in the specimen (body sample) can be determined in a single experiment. In particular, this detection system can also be used to carry out statistically reliable studies using arbitrarily large groups of patients and if appropriate correlations existing between specific diseases and the activity of specific endogenous or exogenous retroviruses can be established therefrom. In the event of a proven correlation between a specific retroviral expression pattern and a specific disease, this test system can also be used for the early identification or to assess the personal genetic risk for such a disease.

[0029] Another very decisive advantage of the method is that the sequences of the PCR primers (MOP) according to the invention, i.e., the degenerated oligonucleotides of the primer mixtures used to amplify the retrovirus-specific RT gene sections to be identified in the specimen do not overlap with the sequences of the synthetic virus-specific oligomers used as dot blot probes. The PCR primer sequences contribute approximately half the final amplificate length. If these sequence sections were also contained in the dot blot probes (cf. Herrmann and Kalden, 1994), this would lead to considerable restrictions in the prediction capability of the test since the amplificates would then hybridise to a certain extent with all dot blot probes on the filter membrane. It is an advantage of the method according to the invention that this undesirable

effect is impeded by using synthetic, precisely defined, homogenous oligonucleotide preparations for both the forward and reverse primers and for the RDBH probes.

[0030] The nucleotide sequences of all exogenous and endogenous retroviruses characterised so far (HERV) are published in gene banks. From these suitable nucleotide sequences can be derived for the synthesis of virus-specific oligomers as dot blot probes. In principle, corresponding oligomers of all these sequences can be dotted onto a single filter membrane. Consequently, it is possible to test a specimen for the entire spectrum of retroviruses known so far in a single experiment. Compared with the prior art whereby its own diagnostic test, especially a serological test using a special antibody, must be carried out to identify each putative virus in a sample, the method according to the invention thus represents significant progress.

[0031] As a result of the known higher sensitivity of PCR and the possibility of repeated amplification of PCR products, the method according to the invention also attains a detection limit which is barely achieved by any other test system.

[0032] In a preferred embodiment of the method according to the invention the "head" or extension section of the primer nucleotides according to the invention consists of the nucleotide sequence GAAGGATCC whereby the nucleotide series GAA is a so-called 'clamp' and the nucleotide series GGATCC represents the interface for the restriction enzyme BamHI. The head or extension sequence has proved very good in practice. In principle, however, the head sequence can also consist of any arbitrary nucleotide sequence provided that the primer annealing kinetics is not negatively influenced.

[0033] The nucleotide sequences of the synthetic oligonucleotide of the RDBH probes are preferably selected so that they correspond to the retroviral nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L or to a section of this region i.e., they match and/or (experimentally) can hybridise with it.

[0034] Since the efficiency of an oligonucleotide synthesis decreases with the length of the oligonucleotide to be synthesised, a variant of the method

according to the invention is provided in which a mixture of equimolar quantities of two comparatively short-chained synthetic oligonucleotides is used as immobilised RDBH probes in each case (i.e. for each probe or each dot). These correspond together or one after the other to a longer, preferably approximately 90 base pairs (bp) long, section of the nucleic acid region of the reverse transcriptase gene between the highly conserved motifs V L P Q G and Y M/V D D I/V/L L.

[0035] In an embodiment that has proved very useful in practice, these two short-chain oligonucleotides are approximately the same size or the same length and preferably comprise approximately 45 base pairs.

[0036] The invention is explained subsequently in greater detail with reference to examples of embodiment and relevant drawings and tables.

[0037] The abbreviations used are as follows:

BaEV = baboon endogenous retrovirus;
ERV = endogenous retrovirus;
ERV9 = endogenous retrovirus type 9.
GaLV = gibbon ape leukaemia virus;
HERV = human endogenous retrovirus;
HIV = human immunodeficiency virus;
HML = human mouse mammary tumour virus-like sequence;
HPLC = High Performance Liquid Chromatography
HRV5 = human (exogenous) retrovirus type 5;
HTLV-1 = human adult T-cell leukaemia virus type 1;
LINE = long, disperse (scattered) DNA sequence element;
MMTV = mouse mammary tumour virus;
MoMuLV = Moloney mouse leukaemia virus;
MOP = Primer mixture of degenerated oligonucleotides comprising forward and reverse primers
MPMV = Mason Pfizer monkey virus;
PCR = polymerase chain reaction;
PERV = porcine endogenous retrovirus;
PBMNC = peripheral blood mononuclear cells;
RDBH = reverse dot blot hybridisation;
RT = reverse transcriptase.

[0038] Table 1: Retrovirus-type ABD and type C specific primer mixtures MOP-ABD and MOP-C according to the invention which each contain forward and reverse primers and comprise degenerated oligonucleotides. The standardised single-letter abbreviation code of the IUPAC nomenclature has been used to describe the degenerated oligonucleotide sequences (see *European Journal of Biochemistry* 150: 15, 1985). Both the forward primer and the reverse primers are shown in the 5'-3' direction following the IUPAC conventions and relative to the DNA strand. The degree of degeneration, in other words the number of different specific embodiments of this primer that can be obtained by synthesis, is given in each case in the form of the theoretically calculated number of different oligonucleotides.

[0039] Table 2: Immobilised synthetic retrovirus-specific oligonucleotide probes for producing dot blot membranes. A mixture of equimolar quantities of both partners of an oligonucleotide pair which corresponds to a 90 bp long section of a retrovirus-specific reverse transcriptase was prepared for each spot or dot. In each case 100 picomole of these mixtures was applied to the membrane in the configuration corresponding to the code shown. A dilution series of human genomic DNA (8E-8H) and oligonucleotide primer mixtures (8I-8L) was applied to the filter for internal standardisation of the hybridisation and autoradiography. For each oligonucleotide sequence used on the filter the gene bank access number and the first author are given where available.

[0040] Table 3: Classification of retrovirus-specific oligonucleotide dot blot probes: From 61 representative members of all known human exogenous and endogenous retroviruses the nucleotide sequence of the appropriate reverse transcriptase gene in the region between the highly conserved domains V L P Q G and Y M/V D D I/V/L L was used to synthesise dot blot probes (Shih et al. 1989, Donehower et al. 1990). In the experiment shown here 21 retroviral nucleotide sequences from type ABD (HERV-K superfamily), 19 retroviral nucleotide sequences from type C, 1 retroviral nucleotide sequence from type D and 7 nucleotide sequences related to the human foamy virus were used. Also tested were a human LINE-1 sequence (3L) and 6 exogenous human retroviruses (6E-6J), as well as five probes which correspond to a mammal C type retrovirus and a probe which corresponds to a mammal B type retrovirus (7E-7J).

[0041] Asterisks indicate nucleotide sequences related to HERV transcripts which were found in patients with multiple sclerosis and patients with systemic lupus erythematosus.

[0042] FIG. 1 Localisation of conserved amino acid sections in the amino-terminal gene regions of the reverse transcriptase of retroviruses and retrotransposons. The core homology regions V L P Q G and Y M/V D D I/V/L L were used to derive and prepare the degenerated oligonucleotides of the primer mixtures according to the invention (MOP-ABD or MOP-C).

[0043] FIG. 2. Schematic of the RT-PCR/RDBH method according to the invention.

[0044] FIG. 3. HERV expression pattern in human PBMNCs of a healthy blood donor.

[0045] The reverse dot blot hybridisation (RBDH) was carried out under standard conditions using DNA fragments which had been amplified using the primer mixtures of degenerated oligonucleotides according to the invention, namely MOP-ABD (Table A) or MOP-C (Table B) or the combination MOP-ABD/MOP-C (Table C).

[0046] FIG. 4. HERV expression in human PBMNCs after adding a cloned DNA fragment that contains a PERV RT gene. Less than 10 copies of a porcine endogenous retrovirus (PERV) type A DNA (Patience et al. 1997) could be detected and identified under standardised test conditions (Table A, filter code 7F). Under the stringent conditions used no cross hybridisation of HERVs with porcine-specific amplification products obtained from a porcine DNA matrix were observed (Table B).

[0047] Example 1: RNA preparation

[0048] Total RNA was extracted from peripheral blood mononuclear cells of a healthy blood donor using the guanidine isothiocyanate/caesium chloride (GIT/CsCl) ultracentrifugation protocol proposed by Sambrook et al. (1989) and dissolved in distilled water treated with diethylpyrocarbonate (DEPC). The

mRNA was then enriched using conventional methods e.g. using the commercially available enrichment kit 'Dynabeads™ paramagnetic particles' according to the manufacturer's instructions (Dynal, Hamburg, Germany). The nucleic acid concentration was determined by means of spectrometry at 260 nm. In order to check for any contamination with genomic DNA, 50 ng of each mRNA preparation was used directly, i.e., without first having undergone a reverse transcription, in a polymerase chain reaction (PCR) using the primer mixtures of degenerated oligonucleotides (MOP) according to the invention. Only those preparations which showed no DNA traces were used for the actual PCR. Those preparation formulations for which some DNA contamination could be detected were treated with 100 units/µg RNase-free DNase (Roche Diagnostics, Mannheim Germany) in 100 mM pH 5.0 sodium acetate, 5 mM MgSO₄, until the control PCR yielded a negative result.

[0049] Example 2: Preparation of MOP-ABD and MOP-C primer mixtures according to the invention for PCR

[0050] Table 1 shows preferred MOP-ABD and MOP-C primer mixtures using the IUPAC nomenclature familiar and commonly used in specialist circles. Each of the primer mixtures contains a plurality of different forward and reverse primers. The forward primers of the MOP-ABD primer mixture exhibit the general nucleotide sequence GAAGGATCCARAGTNYTDYCHCMRGGH which comprises 3456 degenerations, i.e. 3456 different specific nucleotide sequences. The reverse primers of the MOP-ABD primer mixture exhibit the nucleotide sequence GAAGGATCCNWDDMKDTYATCMAYRWA which comprises 27648 degenerations, i.e. 27648 different specific nucleotide sequences. The forward primers of the MOP-C primer mixture are characterised by the general nucleotide sequence GAAGGATCCTKKAMMSKVYTRCYHCARGGG, which comprises 3072 degenerations, i.e. 3072 different specific nucleotide sequences and the reverse primers of the MOP-C primer mixture exhibit the nucleotide sequence GAAGGATCCMDVHDRBMDKYMAYVYAHKKA which comprises 8192 degenerations, i.e. 8192 different specific nucleotide sequences.

[0051] These primer nucleotide sequences correspond to the highly conserved core homology regions V L P Q G and Y M/V D D I/V/L L within the reverse transcriptase (RT) gene of all known endogenous and exogenous

retroviruses (see Fig. 1 and the publications of Xiong and Eickbush 1990, Shih et al. 1989 and Donehower et al. 1990). The beginning of the primer nucleotide sequence at the 5' end of the appropriate retrovirus-specific core-homology region, called the "head", namely the nucleotide series GAAGGATCC is an extension sequence which consists of the so-called "clamp" sequence GAA and the *BamHI* restriction site GGATCC.

[0052] Instead of the "clamp" sequence described here and the *BamHI* restriction site described here, another "clamp" sequence and/or another interface for a restriction enzyme can also be used to produce the "head" or extension sequence of the appropriate primer. Basically however the length of this "head" or extension sequence should not be more than half the total primer length.

[0053] The MOP-ABD primer mixture allows the separate amplification of types A, B and D retrovirus and the MOP-C primer mixture allows the separate amplification of type C retrovirus. Both primer mixtures can easily be combined and thus make it possible to amplify all retrovirus types (A, B, C, and D).

[0054] Example 3: preparation of probes for reverse dot blot hybridisation (RDBH)

[0055] Amino acid sequence comparisons have shown that the coding genes of all retrovirus for the reverse transcriptase and most retroelements exhibit highly conserved core homology regions (Poch et al. 1989, Shih et al. 1989, McClure 1993, Donehower et al. 1990, Xiong and Eickbush 1990). Two of the most conserved amino acid sequence sections are the amino acid motifs V L P Q G and Y Y/M D D I/V/L L (Fig. 1). The sequence region between these motifs comprises approximately 90 base pairs (i.e. is approximately 90 bp long) and exhibits a significantly lower homology within the various retrovirus families. This region was used to prepare retrovirus-specific probes for RDBH.

[0056] Here the following procedure was adopted: first, generally accessible nucleotide sequence data banks were searched for nucleotide sequences related to the nucleotide sequence of the reverse transcriptase (RT). Sequences of exogenous and endogenous retroviruses were classified according to the valid nomenclature and subclassified into subclasses in terms

of their RT homology (data not shown here). Some as yet unpublished HERV sequence data were kindly made available by Martin Herrmann (1998) and some were characterised by ourselves. Representative members were selected from all known retrovirus families (Table 3) and in each case an approximately 90 bp long fragment was isolated from their respective RT gene, in each case in the region between the highly conserved RT motifs V L P Q G and Y M/V D D I/V/L L, and was used as a template for synthesising the corresponding RDBH probes. Since the efficiency of an oligonucleotide synthesis decreases with the length of the oligonucleotide to be synthesised, instead of a 90 pb long oligonucleotide (90-mer) two 45 bp long oligonucleotides (45-mers) were synthesised in each case and used as a pair. Each dot (spot) of the dot blot prepared as described here corresponds to an equimolar mixture of equal proportions of one pair of 45-mers from the group of pairs listed in Table 2.

[0057] Example 4: preparation of reverse dot blot membranes for RDBH

[0058] Retrovirus-specific oligonucleotides which correspond to a 90 bp long fragment of the highly conserved domains of the RT gene were synthesised and purified by HPLC. For each retroviral nucleotide sequence to be tested equimolar quantities of both partners of a pair of 45-mer oligonucleotides prepared as in example 3 were mixed together and 100 picomole of this pair mixture was dissolved in 5x SSC (1x SSC = 0.15M NaCl plus 0.015 M sodium citrate) and then dropped manually or by machine onto a commercially available filter membrane (for example, a ZETApore™ GT blotting membrane supplied by BioRad, Hercules CA USA) using a commercially available dot blot apparatus (for example, Minifold I dot blotter SRC96D made by Schleicher & Schuell, Dassel, Germany). The filters were equilibrated in 2x SSC, the oligonucleotides were irreversibly immobilised, preferably by means of UV cross linking (for example, using the commercially available UV emitter Stratalinker™ supplied by Stratagene, La Jolla, CA USA) and the filters were then dried in air.

[0059] After the amplificate DNA has been hybridised to the RDBH probes covalently linked to the membranes, bound amplificate DNA can be re-dissolved from the dot blot membrane by alkaline denaturation and if necessary re-amplified to achieve sufficient quantities of double-strand DNA, e.g. for cloning and subsequent sequence analysis of the amplificates concerned.

[0060] Example 5: reverse transcription and polymerase chain reaction (RT-PCR)

[0061] From each test formulation 500 ng of DNA-free mRNA were reverse transcribed at 37°C for 1 hour in 50 µl of a solution of 20 mM pH 8.4 tris/HCl, 10 mM dithiothreitol (DTT), 50 mM KCl, 2.5 mM MgCl₂, 0.5 mM of each desoxynucleoside triphosphate (dNTP), 10 units of RNasin (Promega) 30 pmol Random hexamer oligonucleotides (Promega) and 20 units of MLV reverse transcriptase (GIBCO-BRL).

[0062] The formulations were then denatured, for example by heat treatment at 95°C for 5 min and stored at -20°C before further usage.

[0063] For the MOP-PCRs according to the invention (with MOP-ABD and/or MOP-C) in each case a volume of one twentieth (1/20) of the cDNA reaction was amplified in 50 µl of a solution of 10 mM pH 8.3 tris/HCl, 50 mM KCl, 2.5 mM MgCl₂, 0.001 % gelatine, 50 pmoles of the relevant primer mixture(s) of degenerated oligonucleotides according to the invention, 0.25 mM of each desoxynucleoside triphosphate and 1.25 units Taq polymerase (GIBCO-BRL). The test formulations were prepared on ice and coated with 50 µl of mineral oil (Sigma). Amplification was carried out in a commercially available DNA thermal cycler (for example supplied by Perkin Elmer Cetus) using the "Hot-Start method" familiar to the specialist whereby 30 cycles were run through, each having the following parameters: 30 sec at 94°C, 4 min at 50°C and 1 min at 72°C. Finally an extension step was carried out at 72°C for 7 min. The annealing time, i.e. the time taken for double strand formation, was 4 minutes in order to ensure that the vast majority of the primers (degenerated oligonucleotides) contained in the primer mixture according to the invention find the matrices homologous to them. The extension sequence of the primer according to the invention has a stabilising effect on the primer matrix binding kinetics so that the PCR products formed in the first PCR cycle are amplified significantly more efficiently in the following cycles. There is thus the advantage that retroviral matrices are (can be) then amplified themselves if the exactly matching primer is not present in the primer mixture according to the invention. In addition, it is possible to achieve fast cloning of the amplification products e.g. for a sequence examination or to characterise new RT-related nucleotide sequences.

[0064] The reaction conditions for the PCR were optimised with respect to the amount of primer, the annealing time (double strand formation time) and the annealing temperature (double strand formation temperature) in order to achieve an optimum product yield.

[0065] In order to detect product contamination from previous PCR experiments and any traces of genomic DNA contaminations in the solutions used, a control reaction was carried out in which the matrices were omitted. The amplification products were separated electrophoretically on preparative 2.5 % TBE agarose gels and stained with ethyldium bromide. Bands of between 100 and 150 bp which corresponded to the amplified retroviral RT nucleotide sequences were cut from the gel and cleaned using a commercially available cleaning set (for example, the GENECLEAN II kit from BIO 101 Inc., Vista CA USA). For the RDBH approximately 50 ng of the cleaned fragment was labelled with $[\alpha\text{-}^{32}\text{P}]$ dATP (3000 Ci/mmol). The labelling was carried out using a Megaprime DNA labelling kit (Amersham Pharmacia Biotech, England) but can also be carried out equally well using other common labelling methods.

[0066] Example 6: reverse dot blot hybridisation (RDBH)

[0067] The RDBH method was used both to detect and to identify the amplified products. This RDBH method can strictly discriminate (distinguish) PCR products so that any wrongly amplified nucleotide sequences which are not related to nucleotide sequences of retroviral RT genes are of no importance. The high stringency of the RDBH is achieved by using synthetic HERV-specific oligonucleotides according to the invention which are applied as RDBH probes onto the dot blot filter membrane. The important advantage of these RDBH probe oligonucleotides according to the invention is that they contain none of the nucleotide sequences which are exhibited by the degenerated oligonucleotides of the MOP-ABD and MOP-C PRC primer mixtures according to the invention (see, for example, Table 1) and thus differ fundamentally from these PCR primer oligonucleotides. This fundamental difference between the RDBH probe oligonucleotides and the PCR primer oligonucleotides ensures that a hybridisation between an RDBH probe and a PCR amplificate only takes place if the nucleotide sequence between the two primers corresponds to the relevant RDBH probe oligonucleotide, i.e. if this

nucleotide sequence is identical to the relevant RT nucleotide sequence section or only differs in a few nucleotides (n = 3). In the ideal case the hybridising DNA sequences should be completely identical. In practice, under the given hybridisation conditions differences of two to three nucleotides are tolerable. Consequently, under highly stringent conditions even closely related retroviral nucleotide sequences can be distinguished from one another and be uniquely identified.

[0068] In order to avoid cross hybridisations, the optimum stringency conditions for the RDBH were determined by varying hybridisation temperature, washing temperature and salt concentrations. Pre-hybridisation of the reverse D blot filters was carried out in heat-sealed plastic pockets in 0.25 M pH 7.2 Na₂HPO₄, 7 % sodium dodecylsulphate (SDS), 1 mM EDTA at 50°C for at least 3 hours. For the actual hybridisation these solutions were mixed with 5x10⁵ CpM of the labelled PCR amplicate per ml hybridisation volume and incubated for 16 hours under the same conditions. The membranes were then washed twice in 40 mM pH 7.2 Na₂HPO₄, 5 % SDS, 1 mM EDTA and twice in 40 mM Na₂HPO₄ pH 7.2, 1 % SDS, 1 mM EDTA (for approximately 30 min in each case). The reaction was studied and evaluated by autoradiography.

[0069] Example 7: Analysis of the HERV transcription pattern in human PBMNCs using the PCR/RDBH method according to the invention

[0070] First, using the method shown in Fig. 2, total RNA was extracted from human PBMNCs as in Example 1 using common isolation techniques. This total RNA was first subjected to an RT-PCR/RDBH using the MOP-ABD primer mixture according to the invention. Here almost exclusively type B-related HERVs, i.e. members of the HERV-K superfamily were detected (Fig. 3A). The most transcripts originated from members of the HERV-K subgroups HML-2, -3, -4 and -6. Also found were signals from HERV-KC4 related elements (8A, 8B) and from another HERV-K related nucleotide sequence which could not be assigned to any of the HML subgroups (5F). The observed expression pattern agrees with the studies already published which established a differentiated expression of HML elements in human tissue in the result (Medstrand et al. 1993, Andersson et al. 1996). Also found were small quantities of the element HERV-L related to the human foamy virus whereby

the high specificity of the MOP-ABD primer mixture for type ABD related elements is shown.

[0071] At the same time as this test the total RNA from PBMNCs was subjected to an RT-PCR/RDBH using the MOP-C primer mixture according to the invention. Unlike the MOP-ABD primer mixture, the MOP-C primer mixture is not only suitable for priming type-C related nucleotide sequences but also amplifies HERV-K related elements of the HML-2, HML-4 and HML-6 subgroups. A strong expression of HERV-E4-1 related elements (2H and 2I), human foamy virus related HERV-L elements (1E to 1K) and ERV9 related HERVs (4E to 4G and 4I) could be detected. Although the same quantities of radioactively labelled PCR-amplificates were used in all RDBH reactions, the genomic DNA probes present on the membranes (8E to 8H) after hybridisation using MOP-C produced PCR amplificates yielded significantly stronger signals than after hybridisation using MOP-ABD produced PCR amplificates. These findings indicate that the human genome contains significantly more copies of type C related HERV elements than type B related HERV elements.

[0072] In order to detect all retroviral nucleotide sequences in a single experiment the MOP-ABD and MOP-C primer mixtures were used in a combination of equimolar quantities in a PCR/RDBH method according to the invention. This experiment resulted in a predominant amplification of type C related nucleotide sequences whereas the ABD type sequences remained underrepresented (data not given here). For this reason separate PCR methods were carried out using first MOP-ABD primer mixtures and second MOP-C primer mixtures and the purified amplification products of both methods were combined in equal quantities. The RDBH was then carried out using this combination of amplification products. The signal pattern shown in Fig. 3C was obtained which corresponds to the theoretical combination of the signal pattern of the RDBH method using MOP-ABD amplificates as in Fig. 3A on the one hand and using MOP-C amplificates as in Fig. 3B on the other hand. This finding shows that the PCR/RDBH method according to the invention is overwhelmingly well suited especially as a qualitative method of detection.

[0073] Example 8: Proof of the sensitivity of the PCR/RDBH method according to the invention

[0074] In order to check the sensitivity of the PCR/RDBH method according to the invention with reference to the desired practical application in routine diagnostics e.g. to detect or eliminate any interspecies transmission of PERV with xenotransplants, dilution series experiments were carried out using cDNA from human PBMNCs and decreasing concentrations of a cloned DNA fragment that contains a PERV RT-coding region (Takeuchi et al. 1998). Under standardised test conditions even such a small quantity as 10 copies of PERV DNA could be detected in cDNA obtained from 25 ng human PBMNC mRNA (see Fig. 4A, Filter code 7F).

[0075] No cross hybridisations between human specific amplificates and PERV specific RDBH probes were observed (see Fig. 3C, filter code 7F). Even when the PCR/RDBH method was carried out using pure porcine DNA as PCR matrix (Fig. 4B), no cross hybridisations could be detected between the porcine amplificates and the human endogenous or exogenous retroviral nucleotide sequences. These results are a very strong indication of the very high interspecies specificity of the PCR/RDBH method according to the invention.

[0076] The results obtained in the test described here, namely the weak signal obtained with the murine type C retrovirus specific probe (7 I) surprisingly indicate that the porcine genome DNA also contains PERVs homologous to MoMuLV. Further results of this test, namely the observation of a weak signal with human DNA probes (8E, 8F) also surprisingly indicate that even the human genome possibly contains PERV related nucleotide sequences which have no counterpart on the dot blot membrane used and consequently are probably still uncharacterised. These findings clarify the extraordinary advantage of the PCR/RDBH method according to the invention, namely that it is possible to find, isolate and clone as yet unknown DNA fragments.

Table 1

primer	nucleotide sequence	degeneration
MOP-ABD	forward- <u>GAAGGATCC</u> ARAGTNYTDYCHCMRGGH	3456
	reverse- <u>GAAGGATCC</u> NWDDMKDTYATCMAYRWA	27648
MOP-C	forward- <u>GAAGGATC</u> CTKKAMMSKVYTRCYHCARGGG	3072
	reverse - <u>GAAGGATC</u> CMDVHDRBMDKYMAYVYAHKKA	8192

Table 2

Code	Source	Probe	Oligonucleotide sequence (5' ->3')
1A	U35102, Medstrand et al.1993	HML-1	ATGCTAAATAGCCCAACTGTTGTTAAACTTATGTCAGAAAG ATGTTAAATAGCCCAACTATTGTCAAACCTATGTTGGGAAA
1B	S77579, Levebvre et al. 1995	SEQ29	ATGTTAAATAGCCCAACTATTGTCAAACCTATGTTGGGAAA ATTAAGCCAGTTAGAGAACAGTTAAAAATGTTATAGTATT
1E	G895836, Cordonnier et al. 1995	HERV-L	TATATCAACTCTCCGGCTTGTCATAATCTTATTAGAGT CTTGATCACTTTCACTGCCACAAGATATCACACTGGTCCAT
1F	Herrmann 1998	SEQ39	GTATATCAACTCTCCAGCTTGTCATCATCTTATTAGAG CCTTGATCACTTTCACTCTGCAAGATATCATGCTGGTCCA
1G	Herrmann 1998	SEQ40	TTAATCAACTCTCTAGCTTGATCATAATCTTATTGGAGA CCTGATCGCTTTCGCTTCCGCAAGATATCACACTGGGTTT
1H	Herrmann 1998	SEQ45	TATATCAGTTATCTGGCTTGTCAGCTAATCTTATTGGAGA CTAGATAACTTTCACTCCACAAGATATCACACTGGTCCAC
1I	Herrmann 1998	SEQ48	TATATCAACTCTCCAGCTTGTTATAATTATTAGAGAG TTGATCACTTTTGCTTCCACAAGATATCACACTGATTGCCT
1J	Herrmann 1998	SEQ51	TGTATCAACTCTCTGGCTTGTCATAATCTTACCTGGAGA CTTGATCGCTTTCGCTTCCACNAGATATCACACTGGTCCAT
1K	Herrmann 1998	SEQ58	TATATCAACTCTCCAGTTTGTCATAGTCTTAGTCAGAGA CTTGATCACTTTTGCTTCCATAAGACATCACACTGGCCCAT
2A	M14123 Ono et al. 1986	HERV-K10	ATGCTTAATAGTCCAACTATTGTCAGACTTTGTTAGGTCGA CTTCAACCAGTGAGAGAAAAGTTTCAGACTGTTATATTATT
2B	U87592 Zsiros et al.. 1998	SEQ U87592	ATGCTTAATAGTCCAACTATTGTCAGACTTTGTTAGGTCGA CTTCAACCAGTTAGAGAAAAGTTTCAGACTGTTATATCATT
2E	U12970 Haltmeier et al. 1995	pCRTK1	TTTAAAAACTCCCCTACCCCTTTGGGAAGCCCTCCAACA CTTATACCATTCTGAGCCAGTAACCCTCACTGCACTCTCTCG
2F	U12969 Haltmeier et al. 1995	pCRTK6	TTTAAAAATTCCGCCACCCCTTTGGGAAGCCCTCCAACA CTTCTACCATTCTGAGCCAGTCCCCTTAACTGTAACCTTCT
2H	M10976 Repaske et al. 1985	HERVE41	TTCAAGAACTCCCCACCATCTTGGGAGGCCTGGCTCG CCTCCAGAAGTTCCCACCAAGAGACCTAGGCTGCGTGTGCG
2I	Herrmann 1998	SEQ32	TTCAAGAACTCCCTTACTATCTCGGGAGGCTGACTTG TTGCAAAAGTTCCCTGCTAAAGACCTAGGCTATGTCTGCTC

2J	AF026252, Lindeskog et al. 1998	HERV-H	TTCACAGACAGCCCCCATTACTTCAGTCAGGCCAAATTCTTCT TTATCTGTTACCTATCTCCGCATAATTCTCATAAAAACACAC
2K	Herrmann 1998	SEQ61	TTCAGAGACAGCCCCCATTACTTTAGTCAGCTCTTCTCAT CTACTTCTTCCATCCATCTGTTCTCACCTTATTCAATAC
2L	Herrmann 1998	SEQ66	TTCAGAGACAGCCCCCATTACTTTAGTCAGCTCTTCTCAT CTACTTCTTCCATCCATCTGTTCTCACCTTATTCAATATA
3A	U35236, Medstrand et al. 1993	HML-3	ATGTTAACACAGTCCAACAATTGCCAGACTTATATGGGCAAG TTGAACCTACTCTTAAAAAATTTCACAGTGTACATTATTCA
3B	S66676, Kalden und Herrmann, 1993	HERV1 SLE	ATGATAAACACAGTCCAACAATTGCCAGGCATATGTAGGGCA AAWTGAACCTACYTGTAAAAAATTTCAGTGTACATTATTCA
3C	S77583 Lefebvre et al. 1995	RT244	ATGTTAACACAGTCCCACAATTGCCAGTCATATGTGGGCA ATTGAACCTACTCCTTAAAAAATTTCACAGTGTACATTATTCA
3D	Herrmann 1998	SEQ26	CGTGTAAACAGTCCGACTATTGCCAGAAGTATGTGGGG CAATTGAATCTACTCGTAAAAAATTTCACAGTGTACATTAT
3E	U27240 Seifartha et al. 1995	ERV-FRD	TTCAGAGATAGCCCCATTGTTGGCAAGCCTGGCTAG TTTGCAGGACCTAAGTCTTATATGGAAGGGCATCTCCTAC
3F	Herrmann 1998	SEQ46	TTCAGAGATAGCCCCATTGTTGGCAAGCCTGGCTAGA TGCAGGACCTAAGTCTTATATGGGAGGGCATCTCCTACAGA
3H	M92067 Maeda and Kim 1990	HERV-I	TTCATGGATTACCCAACCTTTGGTCAAATTAGAACAA CTAGACAAAGTTCTGTTCCAAAACAATTATGCCTGCTCAA
3I	U27241 Seifartha et al. 1995	HERV-IP (T47D)	TTCACAGACTCCCCTAATATTGGTCAAATTAGAACAA ATTAGAAAAAGTTTCATTCCAGAACAAATATGCCTCTCCA
3J	Herrmann 1998	SEQ65	AGACTCCCCTAATTTGGCAAATTAGAACAGTGT AAAAAGGGTCATCCCAAAGCAAATATGCTGCTAGTACA
3L	McMillan and Singer 1993, M80343	LINE-I	TGCCCTCTCACCACTCCTATTCAACATAGTGTGGAGTT GCCAGGGCAATTAGGCAGGAGAAGGAAATAAGGGTATT
4A	Herrmann 1998	SEQ34	ATGTTAAATGGTCCCACAATTGCCAGACATATGTGGGGCA CTTGAACCTACTCATAAAAATTTCAGTGTACATTATTCA
4B	Herrmann 1998	SEQ42	ATGTTAAACTGTCCAACAATTGTCAGACTATGTAGAACAA ATTGAACCTACTCATAAAAATTTCAGTGTATATTATTCA
4C	Herrmann 1998	SEQ43	ATGTTAACACAGTCCAACAATTGCCAGATGTACGTGGTGCA AATTGAACCTACTGTAAAAAATTGGTGTACATTNTTCN
4E	X57147, La Mantia et al. 1991	ERV9	TTTAGGGATAGCCCTCATCTGTTGGTCAGGCCCTAGCCAA GATCTAGGCCACTTCTCAAGTCCAGGCACTCTGGTCCTCA

4F	Herrmann 1998	SEQ49	TTCAGGGATAGCCCCATCTATTGGTCAGGCATTAGCC CTTGAGCCAGTCTCATACCTGGACACTCTGGCCCTTGGA
4G	Herrmann 1998	SEQ59	TTTAGGGATAGCCCTCATCTGTTGGTCAGGCACAGGCCA ATCTAGTTACTTCTCAAGTCCAGGACTCTGGTGTTCAGT
4H	Herrmann 1998	SEQ60	TTTAGGGACAGCCCTCACTATTGGTCAGGCACCTCAATT ACCTCTCCAGCTACATCTNNCCYNGCATTTGCTTCAG
4I	Herrmann 1998	SEQ63	TTCAGGGATAGCTCCATCTATTGCCAGGCATTAACCCG ACTTAAGCCAGTCTCATACGTGGACACTCTGTCCCTTGGT
4J	Herrmann 1998	SEQ64	TTTAGAGATAGCCCTCACCTGTTGCCAAGCATTGGCCAA TTTAAGTCACTTCTGCACCCAGGTACCCATAATTCTCAATA
4L	AF009668 Blond et al. 1999	HERV-W	TTCAGGGATAGCCCCATCTATTGCCAGGCATTAGCCCA ACTTGAGTCAATTCTCATACCTGGACACTCTGTCCCTCAGT
5A	AF020092 Seifarth et al. 1995	HERV-K (T47D)	CATGCTTAATAGTCCCACTATTGTCAGTATTTGGGGCG GCTTCAACCTGTCAGGGATCAGTTCCCGATGTTACATCG
5B	Herrmann 1998	SEQ05	ATGCTTAATAGTCCCACTATTGTCAGTATTTGGGGCG CTTCAACCTGTCAGGGATCAGTTCCCGATGTTACATCGT
5C	Herrmann 1998	SEQ10	ATGCTTAATAGTCCCACTATTGTCAGTATTTGGGGGTG TTCAACCTGTCAGGGATTCAAGTTCCCGATGTTACATCGTT
5E	U46939 Griffiths et al. 1997	SEQ U46939	ATGACTAACAGCCTGCCATATGCCAGCTATATGTTGACCA GTAGAGCCTGTTGGCAGCAGTGCCAAAAGTACAATT
5F	U39937 Li et al. 1995	U39937	ATGCTTAATAGTCCAATATTGTCAGACTTTGAGGTGCA CTTCAACCAGTTAGAGAAAAGTTTCAGACTGTTATATT
5G	Herrmann 1998	SEQ35	AACCAGTATCAGGAGTTTACAGCCAGGTAGTCAGGAGGAA AGTCATCCTGGTGCAGTGGAAAGGGCATTGGATTAAAGGC CT
5H	Herrmann 1998	SEQ36	AACAATGTTAGAATGGCTCACAGAACTCAGGAAATACTTA GTATTTAATGGTTGTTACATAAGATAACAACCAAGGAACCA
5I	Herrmann 1998	SEQ41	TACCATGGACGACAAGCCTCGTGTACCAAGGCAGTG CAAGCATTGAATGTGATCGTTGAGGGCAGGGTACCGGAA
5J	Herrmann 1998	SEQ77	TGGAAGGGAGGACTTGAGCACATTCTAAATGT GGCTCCTGTAATTTAACACATTGACACATGCTA
6A	U35161 Medstrand et al. 1993	HML-5	ATGCTAACAGCCTACCATGTGTCAGTAACATGAAATCAA TTGCTCCCCAGTAGAAAATAATTCTAATTGCAAGATTATT

6E	HRU46939 Griffiths et al. 1997	HRV5	ATGACTAACAGTCCTGCCATATGCCAGCTATATGTTGACCA GTAGAGCCTGTTGGCAGCAGTGCCAAAAAGTACAAATTT
6F	Y07725	Foamy virus	TTTTAAATAGTCCAGCATTGTTACAGCTGATGTAGTAGAT CTAAAAGAAATCCCTAATGTACAAGTGTATGTTGATGATATA
6G	Tuke et al. 1997	HTLV1	GTTAAAAATAGTCCCACCCCTGTTGAAATGCAGCTGGCCC CCTGCAGCCCATTGGCAAGCTTCCCCAATGCACTATT
6H	M10060, Shimotohno et al. 1985	HTLV2	GTTAAAAACAGCCCCACCCCTTCGAACAACAATTAGCAG CCTCAACCCCATTGAGGAAAATGTTCCACATGACCATTG
6I	Tuke et al. 1997	HIV1	ATGGAAAGGATCACCAAGCAATTCCAAAGTAGCATGACAA CTTAGAGCCTTTAAAAACAAAATCCAGACATAGTTATCTA
6J	J04542	HIV2	TGGAAAGGATCACCAAGCAATTTCATTGATGAGGCA TTAGAACCTTCAGAAAAGCAAACCCAGACGTCATTCTCATC
7A	U60269 Medstrand et al. 1997	HML-6	ATGCTAACAGTCTACGCTATGTCAGCATTGAGGACAG TTAAAGAAGCCTCGGAATATGTTCCACTGCTTACATCATT
7B	Herrmann 1998	SEQ38	ATGCTAACACCTACGTTAAGTCAGCATTGAGGAGAG AAAGGACTCTCAGAATATGTTCCACTGCCTACATGTTCA
7C	Herrmann 1998	SEQ56	ATGCTAACAGCATTATATCAGCATGTTGAGGATAGGCAT TAAA GGTGCCTCTGAATATGTTCCACAGCCTACATCCGTCTT
7E	M15122 Moore et al. 1987	MMTV	ATGAAAAATAGCCCTACTTATGTCAAAAATTGAGGACAAA ATATTGACTGTAAGGGATAAATACCAAGACTCATATATTGTG
7F	AF038600 Akiyoshi et al. 1998	PERV	TTCAAGAACTCCCCGACCATCTTGACGAAGCCCTACACAG CTGGCCAACCTTCAGGATCCAACACCCCTCAGGTGACCCCTCTG
7G	D10032 Kato et al. 1987	BaEV	TTCAAAAACCTCTCCACTCTTCGATGAGGCTCTCCACAG CTCACCGACTCCGGACCCAGCATCCAGAAGTGACCCCTGC G
7H	M26927 Delassus et al. 1989	GaLV	TTCAAGAACTCTCCACTCTTCGACGAGGCCCTCCACCG TTGGCTCCCTTAGGGCCCTCAACCCCCAGGTGGTGTACT
7I	J02255 Van Beveren et al. 1981	MoMuLV	TTCAAAAACAGTCCCACCCCTGTTGATGAGGCACTGCACAG CTAGCAGACTCCGGATCCAGCACCCAGACTTGATCCTGCT
7J	M12349 Sonigo et al. 1986	MPMV	ATGGCCAACAGTCCTACCTTATGTCAAAAATATGAGGCCAC ATACATAAGGTTAGACATGCCTGGAAACAAATGTATATTATA
8A	U07856, Dangel et al. 1994	HERV-KC4	ATGTTAAATAGTCCCACAGTTGTCAAACCTTGTAGGCAGA ATCCAGCCTGTTAGAGATCAGTTCCAGATTGTGCAGCAA

8B	Herrmann 1998	SEQ31	ATGTTAACAGTTCCACAGTTGTCAAACTTGTAGGCAA ATCCAGCTAGTTAGAGATCAATTCCAGATTGTTACATCATT
8E-8H	human genomic DNA	internal control	100 ng 10 ng 1 ng 0.1 ng
8I-8L	mixed oligo primers	internal control	100 pmol 10 pmol 1 pmol 0.1 pmol

Table 3.

A. Human endogenous retroviral sequences

type-B retroviruses (HERV-K-superfamily)	HML-1 subgroup	HML1 (1A) Seq29 (1B*)
	HML-2 subgroup	HERV-K10 (2A) U87592 (2B)
	HML-3 subgroup	HML-3 (3A) S66676 (3B*) RT244 (3C*) Seq26 (3D*) Seq34 (4A*) Seq42 (4B*) Seq43 (4C*)
	HML-4 subgroup	HERV-K-T47D (5A) Seq05 (5B) Seq10 (5C)
	HML-5 subgroup	HML-5 (6A)
	HML-6 subgroup	HML-6 (7A) Seq38 (7B) Seq56 (7C)
	KC4 subgroup	HERV-K-C4 (8A) Seq31 (8B)
	not defined	U39937 (5F)
type-C retroviruses	HERV-H & related	AF026252 (2J) Seq61 (2K) Seq66 (2L)
	ERV9 & related	ERV9 (4E) Seq49 (4F) Seq59 (4G) Seq60 (4H) Seq63 (4I) Seq64 (4J)

	ERV-FRD	ERV-FRD (3E) Seq46 (3F)
	HERV-ERI family	HERV-E(4-1) (2H) Seq32 (2I)
	HERV-I & related	HERV-I (3H) HERV-IP-T47D (3I) Seq65 (3J)
	HERV-T	S71 pCRTK1 (2E) S71 pCRTK2 (2F)
	HERV-W	AF009668 (4L)
type-D retroviruses	MPMV related	Seq36 (5H)

Table 3 (continued)

Foamy virus related	HERV-L & related	G895836 (1E) Seq39 (1F) Seq40 (1G) Seq45 (1H) Seq48 (1I) Seq51 (1J) Seq58 (1K)
undefined retroviral elements		U46939 (5E) Seq35 (5G) Seq41 (5I) Seq77 (5J)
human non-viral retroposons		LINE-1 (3L)

B. Exogenous retroviruses

Human exogenous retroviruses	HRV5 (6E) Foamy virus (6F) HTLV-1 (6G) HTLV-2 (6H) HIV-1 (6I) HIV-2 (6J)
endogenous Mammalia retroviruses	MMTV (7E) PERV (7F) BaEV (7G) GaLV (7H) MoMuLV (7I) MPMV (7J)